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14. ABSTRACT The highly organized architecture of cells in normal epithelial organs such as the mammary glands is lost in cancer cells. Both the normal as well as the neoplastic phenotypes can be recapitulated in 3- dimensional cell cultures <i>in vitro</i> . Our goal was to revert transformed breast cancer cell lines <i>in vitro</i> back to normal and identify the molecular and genetic changes required for this reversal. We have identified the Rho family GTPase Cdc42 and two proteins that regulate the Rho family of proteins, as key molecules in this process. Inhibition of Cdc42 blocks the hyper-proliferation of transformed acini while activation causes partial reversal of polar architecture without blocking hyperproliferation, suggesting that a tight control of Cdc42 function is required for maintaining normal acini. Knocking down of the Cdc42 activator β -Pix also led to a loss of polarity. A knock-down of P190RhoGAP which inhibits Rho was also found to cause polarity loss. Both of the loss of polarity phenotypes could be rescued by growing these acini in natural basement membrane, Matrigel. These findings are consistent with the idea that epithelial transformation requires a combination of genetic as well as extracellular changes.					
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INTRODUCTION:

Epithelial cells in the mammary glands form structures whose basic unit is a polarized monolayer of cells arranged in a tubule or acinus around a lumen. These cells are highly susceptible to neoplastic transformation, which is characterized by the loss of polarity and organized tissue structure.

The structure of normal epithelial tissue can be recapitulated in-vitro, by embedding mammary epithelial cells in a 3-dimensional (3-D) culture matrix comprised of either Matrigel, a purified basement membrane or type I Collagen gels [1]. When the mammary cells are plated in these 3-D environments, they form spherical structures lined by a polarized monolayer of cells with the apical surface facing a single central lumen, thus recreating normal mammary acini in vitro (Figure 1).

Cancer cells in 3-D cultures fail to form these polarized cysts or acini; instead producing solid masses of cells with very small or no lumens. We proposed to take advantage of this characteristic, to screen for mutations in human breast cancer cells that can restore normal acinar architecture.

BODY:

TASK 1. To introduce insertional mutation by Sleeping Beauty (SB) transposon in human breast cancer cells in vitro and isolate normal acini.

The following cells lines were tested in 3-dimensional culture: MCF7, T47-D and BT-474. As positive control, 184B5 normal mammary cells and MDCK epithelial cells were tested since these cell lines are known to be polarized and normal in morphology. MDCK cells treated with the integrin blocking antibody AIIB2 was used as a negative control as this recapitulates tumorigenic phenotype in 3-D.

MCF7, T-47D and BT-474 cell line showed a non-polarized architecture in matrigel cultures (Figure 2) compared to control 184B5 and MDCK cells. Relative to the two control cell lines, MCF7, T-47D and BT-474 had disorganized acinar architecture with nuclei in the center of the acinus (Figure 2 A, B, C). In contrast, 184B5 cells had nuclei organized in a circle at the periphery of the acini and rudimentary central lumens (Figure 2D). MDCK cells showed organized nuclear arrangement similar to 184B5 cells but with a large central lumen (Figure 2E).

Upon carrying out insertional mutagenesis, the cells were plated in 96-well clear bottom plates, coated with 100% matrigel on the bottom layer and 2% matrigel mixed in with the cells and allowed to grow for 10 days. GE InCell Analyzer1000 high throughput analyzer was used to screen 96-well plates containing acini of the respective cell lines. The analyzer was inefficient in identifying mammary acini in matrigel due to the fact that in 3-D cultures, acini are in many different planes of focus and the machine can only scan a limited number of planes. We hence augmented our search with visual inspection

under the microscope (Figure 3A). We were unable using this strategy, to identify acini that reverted back to normal. In mammary acini it was not possible to see a central lumen under phase contrast (Figure 3B,C), lumens were only identifiable when cells were fixed and stained for molecular markers such as Actin and nuclei as in Figure 2D, thus precluding the recovery of viable acini that can be isolated and cultured. The notable exception was the normal epithelial cells MDCK which produced large lumens easily identifiable under a phase contrast microscope (Figure 3D).

We subsequently focused on reverting the transformed phenotype produced by MDCK epithelial acini when its adhesion is interrupted by the integrin function blocking antibody AIIB2. This antibody blocks the cell-matrix adhesion receptor β 1-Integrin. Acini grown in the presence of AIIB2 in 3-D culture of collagen gels are hyper-proliferative and non-polarized, lacking a central lumen and grow to be larger in size compared to normal control acini (Figure 4); in essence exhibiting many of the phenotypic characteristics of tumor cells in 3-D cell culture.

The small GTPase Cdc42 has been found to be hyperactive in a number of mammary tumors and has been shown to regulate Epidermal Growth Factor (EGF) signaling which is a critical regulator of breast cancer progression [2]. We chose to test whether a recently developed inhibitor against Cdc42, Secramine A [3], can block AIIB2 induced cell proliferation. Treating the acini grown in the presence of AIIB2, with Secramine A led to a strong reduction of hyperproliferation (Figure 5) as seen in the decreased number of nuclei in the acinar cluster in the presence of Secramine A (Figure 5B). The observation suggested that inhibition of Cdc42 can block AIIB2 mediated hyper-proliferation of acini.

TASK 2. Identify the genes whose interruption restores normal acinus formation.

The small GTPases Cdc42, Rho and Rac are key regulators of the actin cytoskeleton and microtubules which regulate motility and polarized architecture of cells. The regulated activity of these proteins is critical for normal epithelial cells to establish the highly ordered architecture found in epithelial organs such as the mammary gland. One hallmark of cancer cells is higher motility and loss of polarization. Hence we tested to determine if up-regulation of these proteins in transformed cells can rescue the transformed phenotype back to normal.

Activated forms of GTPases Rac and Cdc42 were introduced into MDCK cells under the control of a tetracycline repressible promoter using the plasmid pADtet. Cells were grown in 3-D culture in the presence of AIIB2 with or without the expression of the transgene. We found that the constitutive activation of Cdc42, partially rescues the tumorigenic phenotype (Figure 6). In the presence of activated Cdc42, AIIB2 treated acini produced multiple central lumens instead of a solid lumenless structure typical of AIIB2 treated acini. The rudimentary lumens were lined by polarity protein podocalyxin

which is present around normal epithelial lumens (Figure 6B). Cdc42 activation and the partial reversal did not inhibit cell proliferation and the partially reverted acini remained normal in size compared to control. When Cdc42 was expressed in the presence of AIIB2, acini with internal lumens increased by 32% (Figure 6E).

TASK 3. Stably alter the gene expression of the parental cells by shRNA induced knock-down and/or transgene expression in order to restore normal acinus formation.

We subsequently chose to study the family of proteins that regulate the Rho GTPases Cdc42, Rac and Rho. The human genome contains 69 GTPase Exchange Factors (GEF's) which activate GTPases, GTPase Activating Proteins (GAP's) which inhibit GTPases, and 3 GDP Dissociation Inhibitors (GDI's) which also serve to maintain Rho GTPases in an inactive state by controlling their membrane localization [4].

We chose to systematically knock down the expression of each of these genes using lentiviral shRNA. The pPRIME lentiviral plasmid system [5], was used. For each gene, 3 separate shRNA's were constructed using an algorithm from the Cold Spring Harbor National Laboratory, RNAi central site (URL: <http://katahdin.cshl.org:9331/Lab/siRNA/RNAi.cgi?type=shRNA>). The shRNA's were cloned into pPRIME vector and lentiviral particles encoding each shRNA were generated by transient transfection into 293FT cells. MDCK cells were stably transduced with the lentiviruses and the stable clones were plated in 3-D cultures in 96-well format (Figure. 7).

Two proteins, β -Pix and P190RhoGAP, that regulate the activity of Rho GTPases, produced abnormal phenotypes when their expressions were stably knocked down. The shRNA sequences used for β -Pix and P190RhoGAP are shown in the Appendix. Figure 8 shows the phenotype of the β -Pix knockdown acini (Figure 8 A, B) compared to control acini which express an shRNA against luciferase (Figure 8 C, D) (P190RhoGAP produced a similar phenotype and is not shown). In 3-D collagen cultures, the acini showed a strongly transformed phenotype. The protein Podocalyxin, which normally localizes to the edge of the apical lumen, now localized to the outer periphery of the acini. The acini also demonstrated disorganized arrangement of nuclei and severely disrupted Actin cytoskeleton (Figure 8A). Strikingly, when the same cells were grown in 3-D matrigel culture, they were completely normal in morphology and produced polarized acini with a single central lumen (Figure 8 B). The results demonstrate that the molecular composition of the culture matrix, in synergy with the shRNA mediated genetic changes, determine acinar morphology.

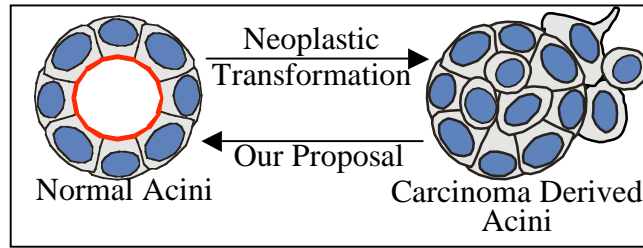


Figure 1: Neoplastic transformation leads to a loss of polarized architecture in epithelial organs as diagrammed above. We propose to identify genetic changes that lead to a rescue of normal morphology.

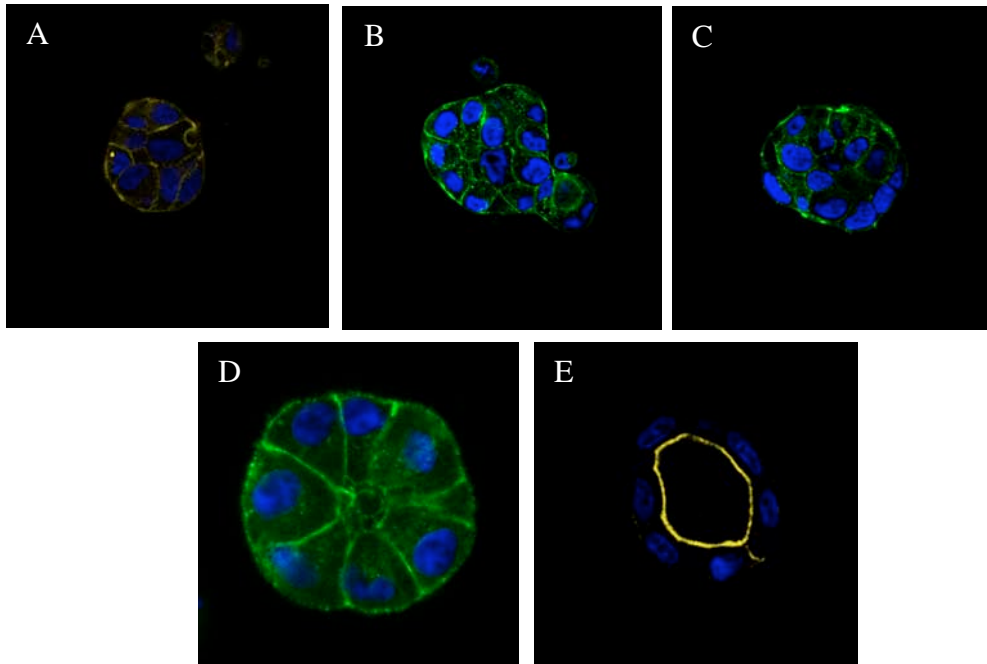


Figure 2: Morphology of epithelial acini in 3-D culture matrix, Matrigel. (A)MCF7, (B) T47-D, (C) BT-474, (D) 184B5 and (E) MDCK acini are shown. Samples are stained for actin (green) and nuclei (blue).

A

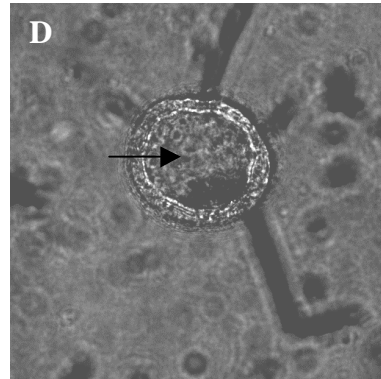
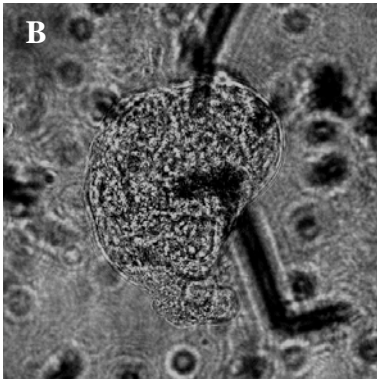
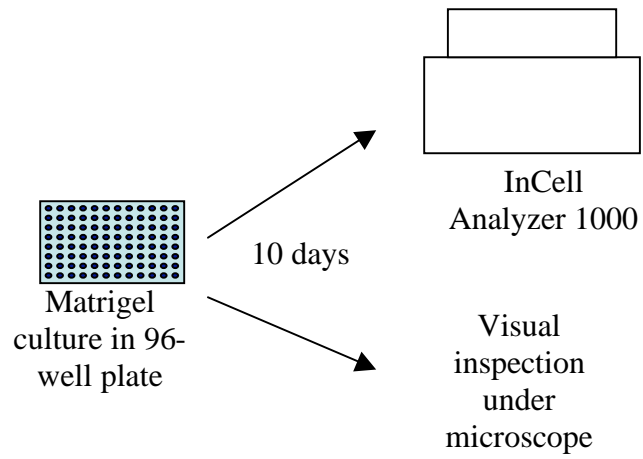


Figure 3: (A) Workflow of screen for indentifying normal acini. Cells were grown in matrigel culture for 10 days followed by fluorescent staining and detection using InCell Analyzer 1000 augmented with visual inspection under the microscope. Identification of acini with lumen under phase contrast. In (B) T47-D and (C) BT474, no lumens can be seen while in MDCK central lumen is clearly visible (arrow) (D).

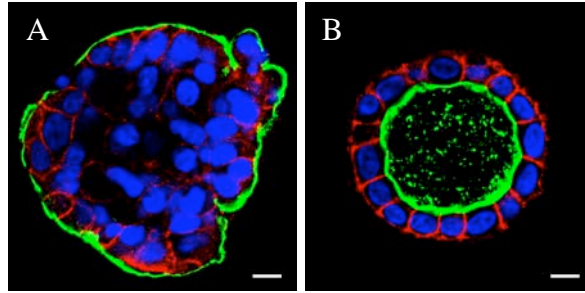


Figure 4: MDCK epithelial cells in 3-dimensional culture treated with AIIB2 produce transformed phenotype (A) compared to normal acini (B). Cells grown in 3-dimensional culture were stained for polarity proteins Podocalyxin (green) and β -Catenin (red) and nuclei (blue).

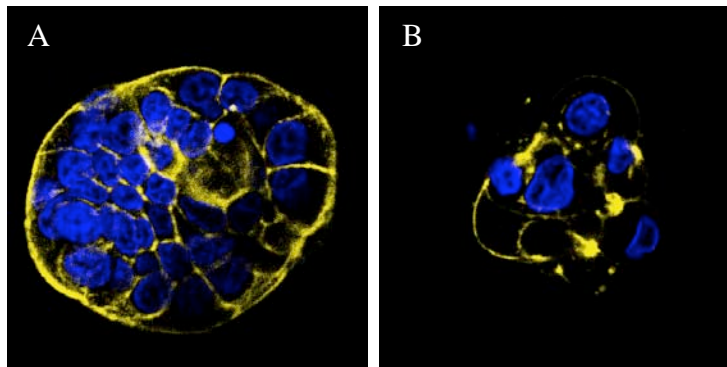


Figure 5: Inhibition of Cdc42 blocks hyper-proliferation of abnormal acini. (A) Acini treated with AIIB2 show hyperproliferation, (B) Acini treated with AIIB2 in the presence of Cdc42 inhibitor Secramine A (2 μ M). Cells were stained for Actin (green) and Nuclei (blue).

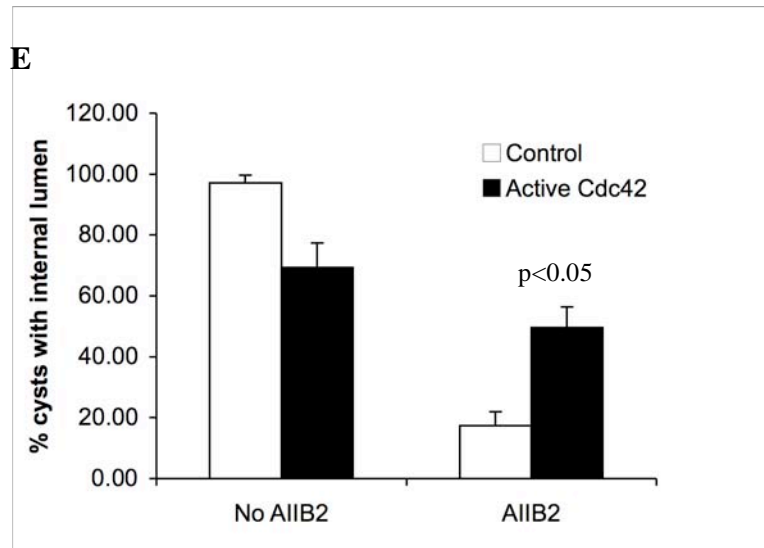
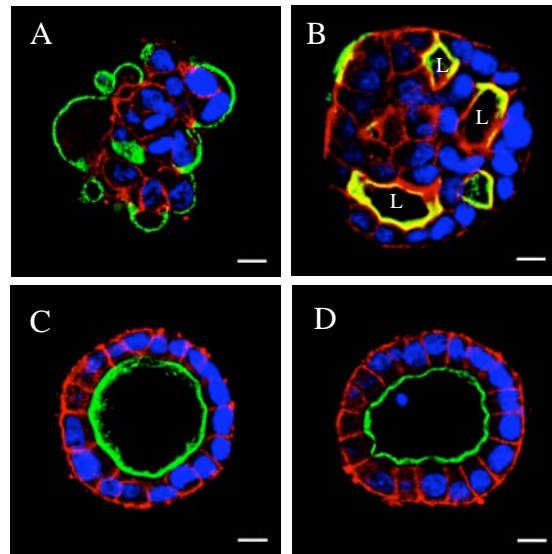


Figure 6: Partial rescue of transformed phenotype by the expression of activated form of Cdc42. Cells grown in 3-dimensional culture were stained for polarity proteins Podocalyxin (green) and β -Catenin (red) and nuclei (blue). (A) Acini grown in the presense of AIIB2, (B) Acini expressing activated Cdc42 in the presence of AIIB2. Rudimentary lumens are labeled 'L'. (C) Control untreated acini. (D) Acini expressing activated Cdc42 alone. (E) Bar graph showing percentage of cyst with internal lumens in cells in the presence of AIIB2 in the presence or absence of activated Cdc42 (Cdc42V12).

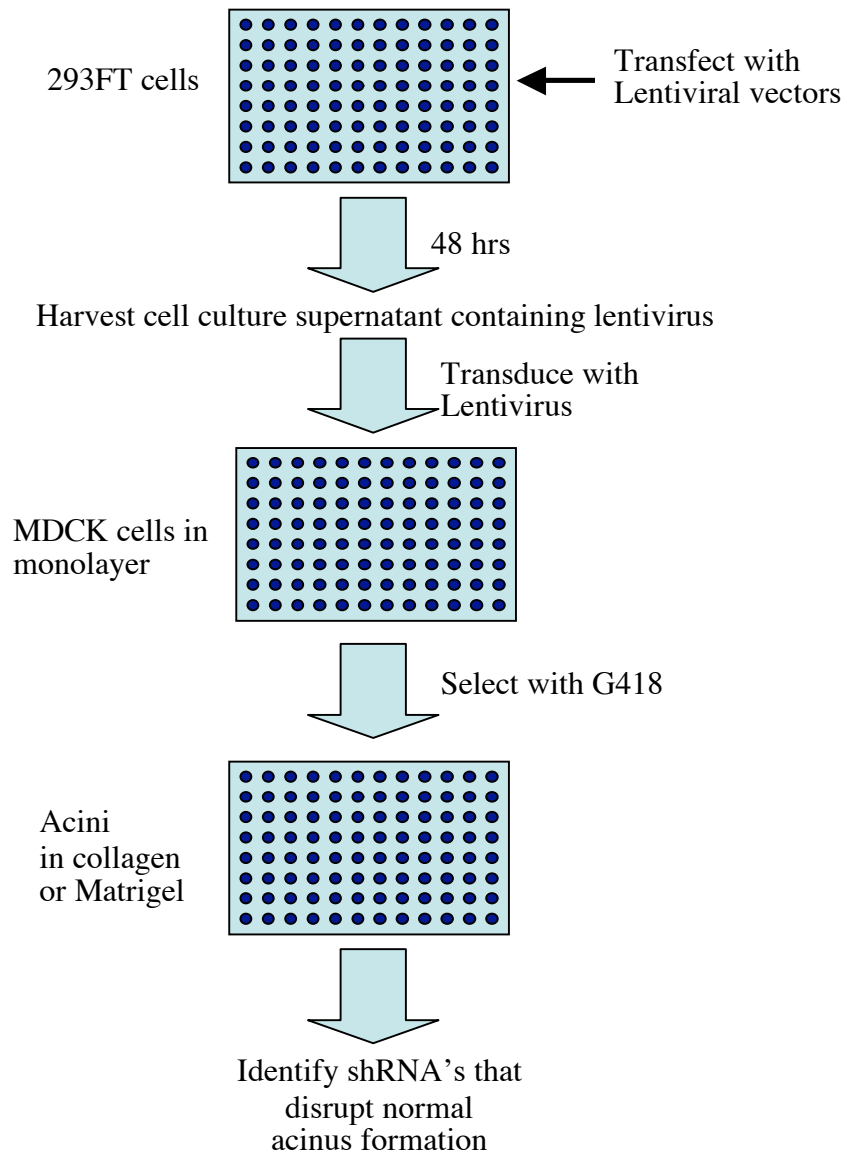


Figure 7: Method of stably knocking down genes with lentiviral shRNA followed by testing for transformed phenotype in 3-D culture.

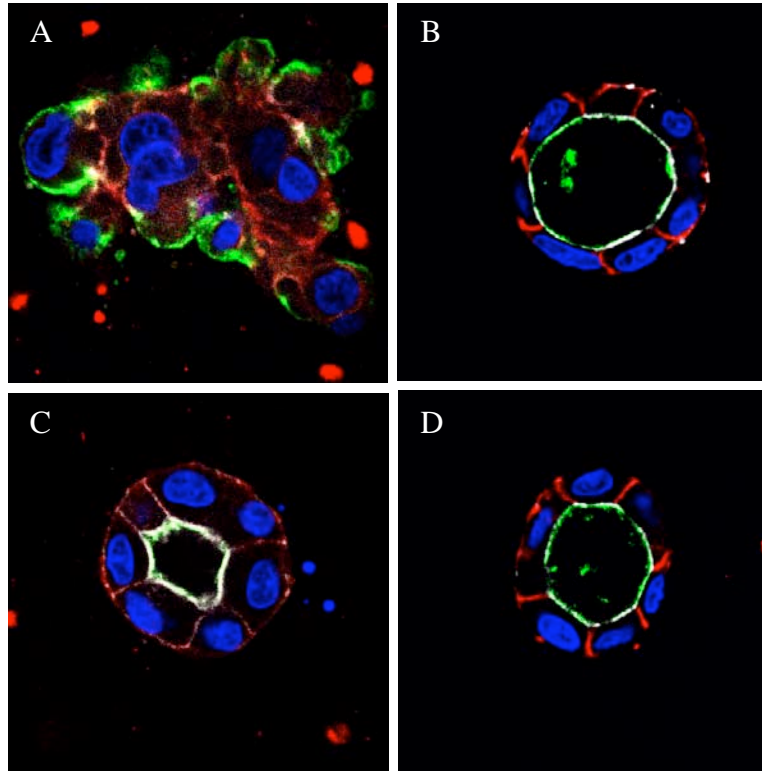


Figure 8: Loss of polarity of β -Pix knock-down cells in collagen and rescue in matrigel. (A) Beta-Pix knockdown cells in 3-D collagen, and (B) in matrigel. Luciferase shRNA expressing negative control acini in (C) collagen and in (D) matrigel. Acini are stained for GP135 (green), β -catenin (red), nuclei (blue) and Actin (white).

KEY RESEARCH ACCOMPLISHMENTS

- Identified active Cdc42 GTPase protein as a putative suppressor of neoplastic transformation
- Demonstrated that the loss of β -Pix GTPase exchange factor gene leads to transformed phenotype in collagen I matrix
- Demonstrated that the loss of P190RhoGAP GTPase activating protein gene leads to transformed phenotype in collagen I matrix
- Discovered that 3-dimensional culture matrix comprised of natural basement membrane (Matrigel) can completely rescue the transformed phenotype produced by the loss of β -Pix and P190RhoGAP

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations: None

Patents and licenses applied for and/or issued: None

Development of cell lines, tissue or serum repositories: None

Informatics such as databases and animal models: Not Applicable

Funding applied for based on work supported by this award: None

Employment or research opportunities applied for and/or received based on experience/training supported by this award: None

CONCLUSIONS:

Reversal of acini formed from cancer cell lines back to normal acini may not be suitable as a screening modality:

We screened several mammary carcinoma cell lines in 3-dimensional matrigel cultures with the goal of identifying acini that revert back to normal in morphology. The idea in this case was to identify the genetic changes that took place due to the insertional mutation that led to a the reversal to normal morphology. Using this strategy we were not able to identify any acini that reverted back to normal. Normal mammary acini such as 184B5 do not produce large lumens that can be seen under phase contrast to identify and isolate live acini for culturing, thus making it extremely difficult to identify reverted cysts that can be isolated..

There are also some possible biological explanations for why such a reversal of morphology to normal may not be feasible. These cells are aneuploid in nature and have accumulated multiple chromosomal aberrations over time in tissue culture that are unrelated to the original cancer and probably necessary for immortalization in culture. Genomic instability in tissue culture that is likely to preclude the ability of these cells to stably revert to normal even if we could identify acini that reverted.

MDCK cell system has several key advantages. MDCK acini produce large visible lumens where phenotypic reversal is more easily identifiable, leaving far less room for ambiguity. These cells also produce acini in 3-D culture much more rapidly than 184B5, allowing for a faster time to obtaining results.

An MDCK 3-D model system that recapitulates cancer:

MDCK cell system is also known to produce tumor like disorganized cell clusters in 3-D cell culture when treated with the antibody AIIB2 [6]. The blockade of β 1-integrin leads to the inhibition of an essential cellular signaling cascade that orients the polarity of the acini. In the absence of this cascade, cells grow in disorganized clusters similar to tumor cells (Figure 4). They also grow to be larger in size, suggesting that the loss of polarity leads to a loss of growth inhibition seen in normal epithelial acini, which stop growing beyond a certain size. Studying this system as a model for tumorigenesis has led us to identify multiple regulators of neoplastic transformation.

Rho GTPases and cancer:

There has been great progress in furthering our understanding of the integrin signaling cascade and its role in cancer [7]. Rho family of small GTPases, are key regulators of cell motility, polarity as well as proliferation. A few key members Cdc42, Rac and Rho, have been found to be hyperactive in breast cancer [8]. Specifically, Cdc42 stabilizes Erb1 and 2 on the cell surface and prolongs Epidermal Growth Factor (EGF) receptor signaling, which is a key contributor to breast cancer progression [9]. Our studies show that acute functional inhibition of Cdc42 in tumorigenic cysts leads to a reduction in acinar size, indicating that Cdc42 is required for the hyperproliferation of AIIB2 treated cysts (Figure 5).

The expression of activated Cdc42 alone led to the production of cyst with normal polarity, however, when activated Cdc42 was expressed in MDCK treated with AIIB2, the acini now developed rudimentary lumens (Figure 6). The evidence suggests that a tightly controlled regulation of Cdc42 is necessary for the formation of normal polarized acini. Activated Cdc42 was sufficient for the formation of multiple small lumens. However, the formation of one central lumen requires the coalition of all the lumens, a process that is likely dependent on a tight control of Cdc42 function.

Studying regulators of Rho GTPases provides insight into the molecular mechanism of reverting cancer:

The function of Rho GTPases are tightly controlled spatially and temporally by three large family of regulators, The GEF's, GAP's and GDI's. It is important to identify which of these regulators are involved in Cdc42 induced neoplastic transformation. Hence we systematically knocked-down the expression of each of these genes using lentiviral shRNA. The search led to the identification of two key genes which regulate Rho GTPase function during transformation-induced loss of polarization.

β -Pix is a GTPase exchange factor with specificity towards Cdc42 and Rac1. β -Pix has been implicated in EGF receptor signaling. Cdc42- β -Pix interaction stabilizes EGF receptor on the cell surface and can lead to uncontrolled cell growth. The striking loss of polarity that we find in cells that have lost β -Pix (Figure 8) suggests that localized Cdc42 activation by β -Pix is critical for the polarity establishment. In addition, we found that the loss of p190RhoGAP leads to a similar loss of polarity, suggesting a role of RhoA in polarity disregulation during acinar formation. Furthermore, for both β -Pix and p190RhoGAP, the loss of polarity can be restored back to normal when these cells are cultured in natural basement membrane matrigel. The data indicates that genetic change alone is not sufficient to cause abnormal polarity. Cell adhesion to the extracellular matrix plays an essential role in determining whether the genetic changes will lead to polarity loss and hyperproliferation. The idea that a combination of both genetic and epigenetic factors are involved in the establishment and maintenance of cellular polarity have been established in previous studies [10]. Here we have identified two of the key genetic changes that when accompanied by the absence of a proper tissue microenvironment, lead to loss of polarity and a transformed phenotype.

Medical applications of this study (So what section):

The loss of Cdc42 activity led to an inhibition of hyperproliferation in tumorigenic acini. Considering the importance of Cdc42 in EGF signaling and its critical role in breast cancer, a Cdc42 inhibitor would be a potentially important breast cancer therapy. This work also identifies β -Pix and p190RhoGAP as key regulators of normal epithelial polarity the loss of which lead to tumorigenic phenotype. This suggests that a drug that activates the function of β -Pix would block tumors. However, in light of the reports that activated β -Pix promotes EGF receptor signaling, a direct inhibitor of Cdc42 rather than β -Pix is likely to be a successful anti-cancer agent. The inhibition of p190RhoGAP leading to polarity and growth deregulation is consistent with our understanding of cancer. RhoA has been shown to be overexpressed in many breast, colon, lung testicular

and squamous cell cancers [11]. Since p190RhoGAP blocks RhoA function, our findings further establish the potential of RhoA inhibitors as potent anti-cancer drugs.

REFERENCES:

- 1) Weaver VM, Howlett AR, Langton-Webster B, Petersen OW, Bissell MJ. (1995) The development of a functionally relevant cell culture model of progressive human breast cancer. *Semin Cancer Biol* 6(3):175-84.
- 2) Hirsch DS, Wu WJ. Cdc42: an effector and regulator of ErbB1 as a strategic target in breast cancer therapy. *Expert Rev Anticancer Ther.* 2007 Feb; 7(2):147-57.
- 3) Pelish HE, Peterson JR, Salvarezza SB, Rodriguez-Boulan E, Chen JL, Starnes M, Macia E, Feng Y, Shair MD, Kirchhausen T. Secramine inhibits Cdc42-dependent functions in cells and Cdc42 activation in vitro. *Nat Chem Biol.* 2006 Jan;2(1):39-46.
- 4) Jaffe AB, Hall A. Rho GTPases: biochemistry and biology. *Annu Rev Cell Dev Biol.* 2005;21:247-69.
- 5) Stegmeier F, Hu G, Rickles RJ, Hannon GJ, Elledge SJ. A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. *Proc Natl Acad Sci U S A.* 2005 Sep 13;102(37):13212-7.
- 6) Yu W, Datta A, Leroy P, O'Brien LE, Mak G, Jou TS, Matlin KS, Mostov KE, Zegers MM. Beta1-integrin orients epithelial polarity via Rac1 and laminin. *Mol Biol Cell.* 2005 Feb;16(2):433-45.
- 7) Chrenek MA, Wong P, Weaver VM. Tumour-stromal interactions. Integrins and cell adhesions as modulators of mammary cell survival and transformation. *Breast Cancer Res.* 2001;3(4):224-9.
- 8) Fritz, G., Just, I. & Kaina, B. Rho GTPases are over-expressed in human tumors. *Int. J. Cancer.* 1999; 81, 682-687
- 9) Wu WJ, Tu S, Cerione RA. Activated Cdc42 sequesters c-Cbl and prevents EGF receptor degradation. *Cell.* 2003 Sep 19;114(6):715-25.
- 10) Bissell MJ, Kenny PA, Radisky DC. Microenvironmental regulators of tissue structure and function also regulate tumor induction and progression: the role of extracellular matrix and its degrading enzymes. *Cold Spring Harb Symp Quant Biol.* 2005;70:343-56.
- 11) Sahai E, Marshall CJ. RHO-GTPases and cancer. *Nat Rev Cancer.* 2002 Feb;2(2):133-42.

APPENDIX

ShRNA sequences designed for stably knocking down Rho regulatory proteins β -Pix and p190RhoGAP and p190B. Oligo names represent gene name followed by oligo number (1,2,3). Sequences shown in red represent the sense and antisense strands that encode the RNA hairpin. The sequence in between the red regions encode the loop of the hairpin.

Oligo Name	Sequence (5'-3')
β -Pix_1	TGCTGTTGACAGTGAGCGCGCAGTGAATGTTCTCACAGAGTAGTGAAGCCACAG ATGTACTCTGTGAGAACATTCAGTGCATGCCTACTGCCTCGGA
β -Pix_2	TGCTGTTGACAGTGAGCGAACTCTGACTATGACAGTATATTAGTGAAGCCACAG ATGTAAATATACTGTCATAGTCAGAGTCTGCCTACTGCCTCGGA
β -Pix_3	TGCTGTTGACAGTGAGCGAGCACAGATTCTGAAAGTCATTTAGTGAAGCCACAG ATGTAAATGACTTTCAGAACTCTGTGCGTGCCTACTGCCTCGGA
P190Rho GAP_1	TGCTGTTGACAGTGAGCGCGGCTGTCTCTGCCTTTACAATTAGTGAAGCCACAG ATGTAAATTGTAAAGGCAGAGACAGCCATGCCTACTGCCTCGGA
P190Rho GAP_2	TGCTGTTGACAGTGAGCGCGGCTACTCATATGTACGATAATAGTGAAGCCACAG ATGTATTATCGTACATATGAGTAGCCTTGCCTACTGCCTCGGA
P190Rho GAP_3	TGCTGTTGACAGTGAGCGATTAGTGCAACTATTGATAAATAGTGAAGCCACAG ATGTATTTATCAATGAGTTGCACTAAGTGCCTACTGCCTCGGA